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14. ABSTRACT:

Sporadic breast cancer represents 90% of breast cancer patients. Mutations of both oncogenes and tumor suppressor genes often occur in spontaneous breast cancer. Specifically, tumor suppressor gene activity may be abrogated or decreased in cancer cells. Recently, a putative tumor suppressor gene, DBC2 (Deleted in Breast Cancer), was discovered that appears to be frequently mutated in sporadic breast cancer. DBC2 is suspected to be a tumor suppressor gene important for breast cancer because: 1) DBC2 expression cannot be detected in half of the spontaneous breast cancer tissues and cells tested and 2) wild-type (WT) DBC2 expressed in a breast cancer cell line, T47D, inhibited cellular proliferation while mutated DBC2 expression did not repress growth of the breast cancer cells. These data imply that mutation of DBC2 is important for the development of spontaneous breast cancer. This work serves to investigate the functional role of DBC2 in cells and mice to elucidate the function of DBC2 for tumor suppression. To this end, we have begun targeting the DBC2 allele and are constructing vectors that will express wild type human DBC2 cDNA and altered cDNAs.

15. SUBJECT TERMS
deleted in breast cancer, DBC2, spontaneous breast cancer

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A Mouse Model to Investigate the Role of *DBC2* in Breast Cancer

INTRODUCTION

Breast cancer is a major cause of mortality among women. The American Cancer Society predicts that about one in eight women will be diagnosed with breast cancer (4). Deletion of tumor suppressor genes play an important role in both familial and sporadic breast cancer (1). Hereditary breast cancer is frequently due to a germline heterozygous mutation of either Brca1 or Brca2 (5). Familial breast cancer represents only 10% of total breast cancer cases; and Brca2 is not mutated for spontaneous breast cancer; thus, the etiology for 90% of breast cancer is largely unknown. However, Hamaguchi and colleagues recently discovered a putative tumor suppressor gene, *DBC2* (deleted in breast cancer), that appears to be frequently mutated in sporadic breast cancer (3). The *DBC2* gene lies within a region of human chromosome 8p21. The putative functional domains of *DBC2* include a RAS domain and two protein-protein interaction domains called the BTB/POZ domains. *DBC2* is suspected to be a tumor suppressor gene important for breast cancer because: 1) *DBC2* expression cannot be detected in half of the spontaneous breast cancer tissues and cells tested and 2) wild-type (WT) *DBC2* expressed in a breast cancer cell line, T47D, inhibited cellular proliferation while mutated *DBC2* expression did not repress growth of the breast cancer cells. These data imply that mutation of *DBC2* is important for the development of spontaneous breast cancer. Recent microarray data shows *DBC2* to have an influence on the following pathways: cell-cycle, apoptosis, cytoskeleton, and membrane-trafficking; suggesting a role for *DBC2* in carcinogenesis (7). Furthermore, Wilkins et al. proposed a model in which *DBC2* may function as a tumor suppressor by facilitating the recruitment of proteins and their subsequent degradation by the Cul3 ubiquitin ligase complex (8). However, due to the fact that *DBC2* was just recently discovered, little is known about its function. This work serves to investigate the functional role of *DBC2* in cells and mice to elucidate the function of *DBC2* for tumor suppression.

BODY

Specific Aim 1: Characterize the phenotype of *DBC2*-mutant mouse ES cell and MEF clones

a) Generate *DBC2*-mutant ES cells

b) Generate knock-ins of wild type and altered *DBC2* cDNAs in ES cells
c) Perform a genotoxic screen on wild type and knock-in ES cells
d) Analyze cell cycle checkpoints and apoptosis in knockout MEF

In the past year, I have constructed another targeting vector to mutate *DBC2* in ES cells. Importantly, a positive selection cassette replaces the initiating ATG and some surrounding sequence. This targeting vector is similar to the vector generated the first year with improvements. The *HPRT* (hypoxanthine phosphoribosyltransferase) minigene is commonly used for selection of transfected clones and is unique compared to other selection cassettes in that it's coding sequence is interrupted by splicing sequences and a small intron such that exons 1&2 are separated from exons 3-8 (6). The advantage in using the *HPRT* minigene compared to other cassettes is that one may select for both the presence and absence of HPRT in HAT (hypoxanthine, aminopterin, thymidine) and 6-thioguanine, respectively. However, the affect these splicing sequences within this minigene might have on splicing at the target gene has not been investigated. It is possible that splicing is restricted to the *HPRT* minigene; however, it is also possible that the splice acceptor (SA) preceding *HPRT* exons 3-8 can trap upstream exons in the target gene similar to promoter trap selection cassettes that have been used to randomly disrupt and identify genes (2). It is also possible that the PGK promoter of the *HPRT* minigene can express a transcript that starts from *HPRT* exons 1&2, but then skips *HPRT* exons 3-8, and instead splices into the SA of downstream exons from the target gene. Either possibility would result in transcripts that may code for proteins and impact phenotype in unpredictable ways. In order to prevent aberrant splicing events and potentially undesired proteins that could cause unwanted phenotypes, the HPRT minigene should be removed by site-directed recombination. In the new targeting vector, the selection cassette is flanked by loxP sites that can recombine with each other upon expression of Cre recombinase as shown in figure 1. Importantly, the same selection cassette can then be used to target the second allele of DBC2.

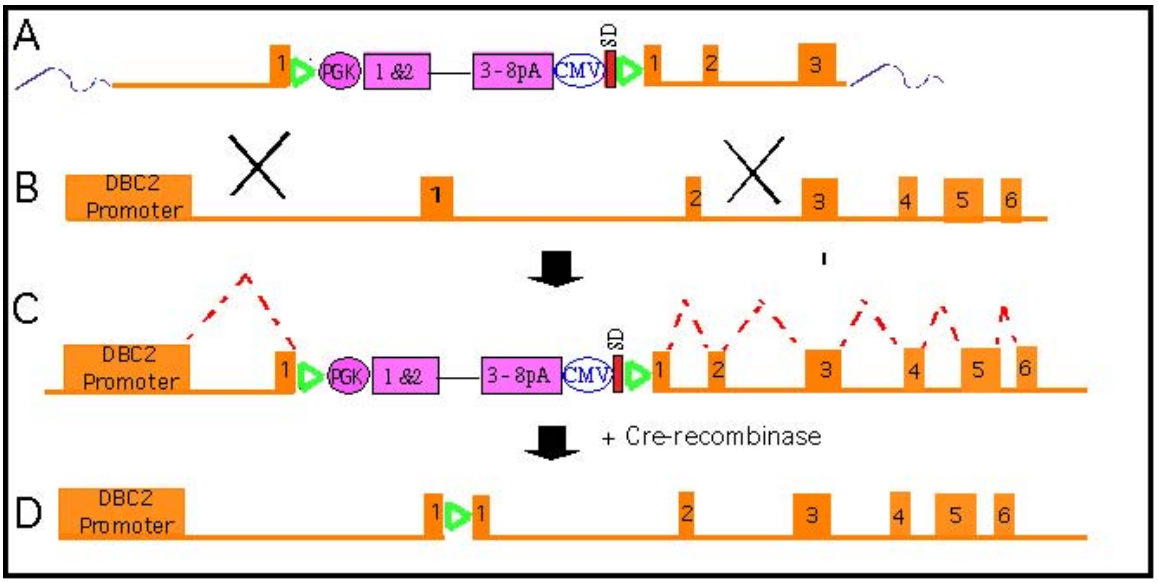
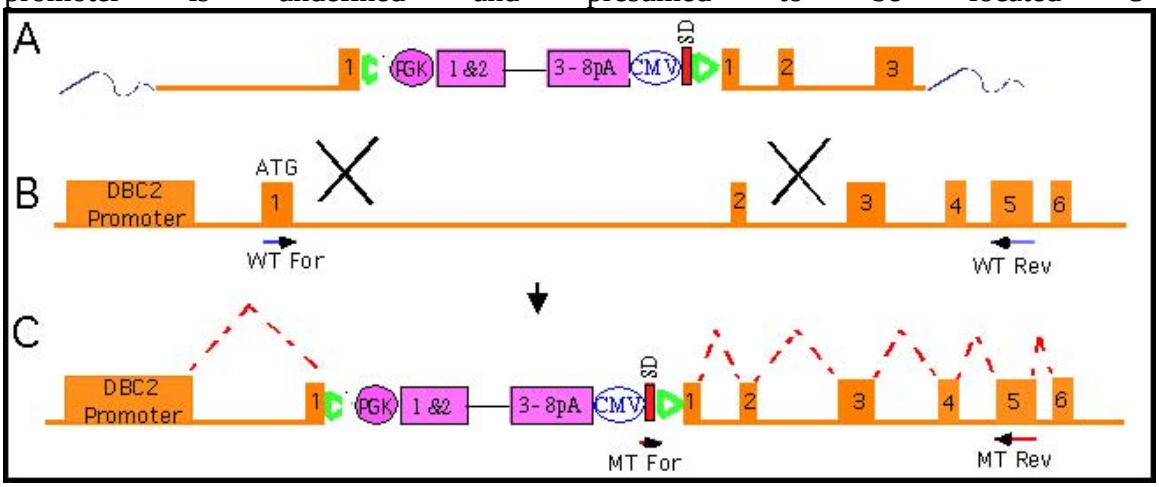


Figure 1. **A**. Gene targeting vector: deletes the translation initiation ATG with an HPRT minigene. **B**. *DBC2* genomic locus. Six exons are shown. The rectangle labeled *DBC2*

promoter is undefined and presumed to be located 5' to the exon that contains the translation initiation ATG. **C**. Insertion of selection cassette into *DBC2* gene. Part of exon 1 is deleted such that the selection cassette replaces the translation initiation ATG. **D**. Removal of selection cassette from DBC2 gene. LoxP sites recombine with each other upon addition of Cre-recombinase deleting selection cassette.

The vector was electroporated into ES cells and I am currently screening for positive clones using the RT-PCR method previously described and summarized below. Figure 2 depicts the screening strategy. This analysis included extracting RNA from the potential targeted ES cell cones, the generation of cDNA, and PCR amplification of the region of interest. Fig. 2 depicts the location of the primers used to amplify both wildtype and mutant *DBC2*. The forward primer used to amplify the wildtype band is situated in exon 1 containing the initiation ATG, the region deleted in the mutant allele (Fig. 2B). Alternatively, the mutant forward primer is positioned 3' to the CMV promoter of the selection cassette in an artificial exon (Fig. 2C). Therefore, this primer should only anneal in a clone targeted for *DBC2*. Both the wildtype and mutant alleles utilize the same reverse primer located in a downstream exon.

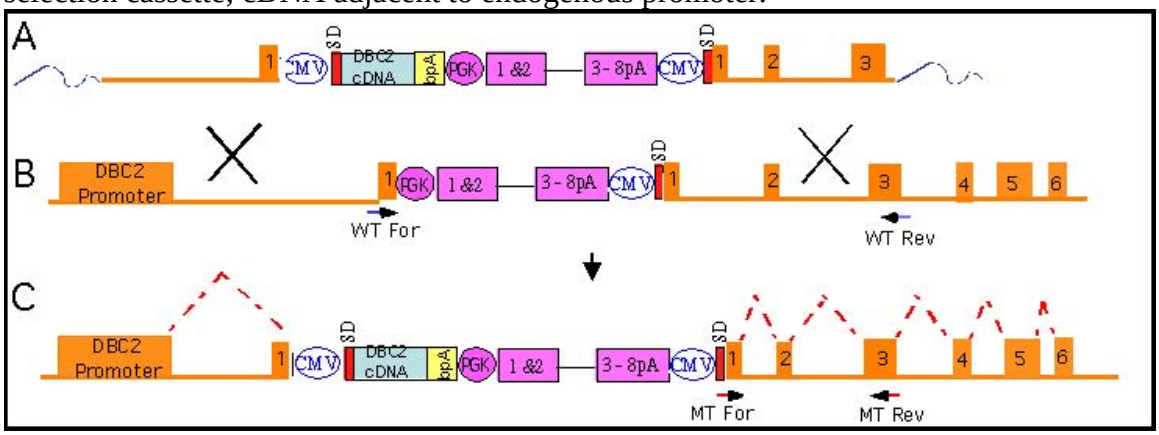
Figure 2. **A**. Gene targeting vector: deletes the translation initiation ATG with an HPRT minigene. **B**. *DBC2* genomic locus. Six exons are shown. The rectangle labeled *DBC2* promoter is undefined and presumed to be located 5' to the exon that contains the translation initiation ATG. WT For and WT Rev show the location of the primers used to identify wildtype *DBC2* by RT-PCR screening. **C**. Insertion of selection cassette into *DBC2* gene. Part of exon 1 is deleted such that the selection cassette replaces the translation initiation ATG. MT For and MT Rev show the location of the primers used to identify the mutant *DBC2* by RT-PCR.



I also began constructing a vector shown in figure 3 designed to facilitate the introduction of cDNA sequences adjacent to the endogenous DBC2 promoter. This vector utilizes homology from the *HPRT* minigene as well as from the endogenous gene to facilitate homologous recombination between the vector and a targeted *DBC2* allele in ES cells. The resulting target allows the cDNA to be expressed by the endogenous *DBC2* promoter. I have successfully amplified and started cloning human *DBC2* cDNA. Once the vector containing Wild type human cDNA is obtained, I will begin making altered

DBC2 cDNAs be introduced adjacent to the genomic *DBC2* promoter. For the altered cDNAs, I will introduce the fragment of cDNA that codes for only the BTB/POZ protein-protein interaction domains. These domains may be dominate-negative. I will also introduce cDNAs mutated for the RAS domain and cDNAs that contain mutations found in breast cancer (3) including the Asp-299 ▸ Asn mutation in the BTB/POZ domain and the Phe-647 ▸ Thr mutation in exon 9 (these two mutations likely inactivate function).

Figure 3. A. Gene targeting vector containing Wild type human *DBC2* cDNA. **B**. Targeted DBC2 genomic locus; *HPRT* minigene deletes translation ATG. **C**. Insertion of cDNA/selection cassette into DBC2 gene; cDNA/selection cassette replace existing selection cassette; cDNA adjacent to endogenous promoter.



Specific Aim 2: Generate and analyze *DBC2* knockout mice

DBC2 knockout mice will be generated and analyzed throughout their entire life span with particular attention given to cancer onset, incidence and spectrum. In addition, in order to investigate any potential tumor suppressor function *DBC2* has for mammary carcinoma in mice, the *DBC2*-mutation will be crossed into transgenic mice predisposed to mammary carcinoma that contain a *neu* proto-oncogene and a dominant negative p53 transgene. The mutant mice will be studied in cohorts of 30 mice (30 *DBC2*+/+, 30 *DBC2*+/-, and 30 *DBC2*-/-).

We will prepare the $DBC2^{+/-}$ ES cells for injection into blastocysts as soon as we obtain targets.

KEY RESEARCH ACCOMPLISHMENTS

- Amplified human DBC2 cDNA

REPORTABLE OUTCOMES

Abstracts:

None.

Manuscripts:

None

Awards: - None.

CONCLUSIONS

During this past year, we have retargeted the *DBC2* gene in mouse embryonic stem cells and are currently screening for targets using the RT-PCR screening approach described previously. Additionally, we have amplified human *DBC2* cDNA and began generating a vector to rescue *DBC2*-mutant cells. Moreover, we will soon begin making altered *DBC2* cDNAs. We will perform a genotoxic screen on wildtype, mutant and cells altered for *DBC2* to help elucidate a function for *DBC2* in ES cells by testing multiple pathways important for chromosomal metabolism and responses to DNA alterations. Our studies will address the novel functional activity of *DBC2* that could have a large impact in our understanding of spontaneous breast cancer development. It is our hope that these studies will help to understand the putative activity of *DBC2* in the cell and elucidate the importance of *DBC2* as a tumor suppressor. This discovery could open many new doors for the development and implementation of drugs for the treatment of breast cancer.

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